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Modulation of testicular macrophage activity by collagenase

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Abstract: Testicular macrophages (TMs) are located in the interstitial tissue of male gonad. These phagocytic cells take part in forming the organ-specific functional blood-testis barrier and participate in the regulation of the local hormonal balance. In the present study, we isolated TMs from testicular tissues using previously described methods - mechanical (M-TMs) or enzymatic, by treatment with collagenase (E-TMs) and then we studied production by these cells of several cytokines and reactive oxygen intermediates (ROI's). Similarly treated oil-induced peritoneal macrophages (PMs) were used as control cells. PMs had a higher baseline level of production of TNF-α, IL-6, IL-10 and IL-12 than M-TMs and collagenase treatment increased the production of these cytokines (except IL-12) by both cell populations. This effect was significantly more expressed in TMs. In contrast to PMs, TMs produced little ROI's when stimulated by zymosan. We conclude that in the case of local inflammation in the testis, ROI-negative TMs do not contribute to the tissue damage and instead may direct the local immune response into humoral pathway.

Key words: Macrophages - Collagenase - Cytokines - Oxygen intermediates - Testis

Introduction

Testicular macrophages (TMs) are located between Leydig cells in the interstitial tissue of male gonad - testis [14]. They play an important role in removing effete cells and testicular apoptotic cells and form a functional barrier additive to natural anatomical blood-testis barrier created by Sertoli cells and myoid cells [7]. TMs may also play a role in gonadotropin hormone storage in the testis by using mannose receptor for storage of lutropin [4, 20] which then, upon demand , is delivered to the neighbouring Leydig cells, the main producers of testosterone in male gonads.

There are several inmmunochemical reports concerning the function of TMs [14, 15], but the problem with TM isolation from testis raises the main barrier for experimental research [5]. Our publication of efficient method of TM isolation and purification allowed to test the immunomodulatory function of these cells. We found that TMs are effective antigen-presenting cells in humoral responses *in vitro* and show a strong tolerogenic activity in the model of delayed hypersensitivity *in vivo* [7]. These studies, although first to show immunogenic potential of TMs, have left many questions unanswered.

The methods of TM isolation either by mechanical shaking or by short collagenase treatment described by us [5] allow to separate TMs from interstitial testicular tissue as adherent and $Fc\gamma R^+$, esterase$^+$ cell population, but enzymatic treatment is much more efficient in comparison to mechanical shaking. On the other hand, enzymatic treatment used for cell isolation significantly changes the cell environment, making it akin to inflammatory area rich in proteolytic enzymes, and thus potentially can influence the cell physiology [9, 13]. The aim of our study was to compare the influence of enzyme (collagenase) treatment of TMs during the isolation procedure versus non-enzymatic isolation of TMs on secretion of several cytokines and release of reactive oxygen intermediates (ROIs). Oil-induced peritoneal macrophages (PMs) treated or not with collagenase served as reference macrophages. Our experiments show that macrophages from both sources, testis or peritoneal cavity, treated *in vitro* with proteolytic enzyme significantly up-regulated the amount of produced IL-6, TNF-α and IL-10.

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Materials and methods

Animals. Inbred CBA/J male mice from our own mouse colony, weighing 22-25 g were used for the experiments.

Reagents. The following reagents were used: collagenase type IA, lucigenin (bis-N-methylacridinum nitrate), zymosan A, o-phenylenediamine, hydrogen peroxide, heparin sodium salt, recombinant murine TNF-α, (Sigma, St. Louis, MO); ethylenediamine-tetraacetic acid (EDTA) (BDH, Poole, UK); RPMI 1640, foetal calf serum (FCS) (Gibco Life Technologies, Grand Island, NY); recombinant mouse IL-6 (PeproTech, Rocky Hill, NY), recombinant

murine IL-12 (Genzyme, Cambridge, MA); monoclonal rat anti-mouse IL-6 (MP5-20F3), biotinylated monoclonal rat anti-mouse IL-6 (MP5-32C11), monoclonal rat anti-mouse IL-12 (C15.6), monoclonal rat anti-mouse TNF-α (G281-2626), biotinylated monoclonal rat anti-mouse TNF-α (MP6-XT3), IL-10 OptEIA^{TM} ELISA Set (all from BD PharMingen, San Diego, CA); biotinylated monoclonal rat anti-mouse IL-12 (C17.8) (Endogen, Woburn, MA); paraffin oil Markol 152 (Exxon Corp., Hutson, TX); horseradish peroxidase streptavidin (Vector Laboratories, Burlingame, CA); pyrogen-free distilled water (Polish-American Institute of Paediatrics, CM UJ, Cracow); granulated milk (Marvel, Chivers Ltd, Coolock, Ireland). For cell cultures Nunc lab ware (Roskilde, DK) was used.

Isolation of macrophages. Peritoneal macrophages (PMs) were flushed out from the peritoneal cavity of mice which 4 days earlier were injected i.p. with 1 ml of Markol 152 mineral oil with 5 ml of phosphate buffered saline (PBS) containing 5 U heparin per ml. These cells contained over 90% macrophages (FcR^{+}- and esterase-positive cells) and were not further purified [17-18]. Aliquot of cells (5×10^{7}) was treated for 10 min with 80 μM of collagenase in PBS adjusted to pH 7.0 and then cells were washed twice in excess PBS containing 20% FCS and resuspended in RPMI 1640 medium supplemented with 5% or 10% FCS. The cell viability, as assessed by trypan blue exclusion test, was >95% in all groups. For further tests the cells were allowed to adhere for 2 hrs at 37˚C in appropriate labware and then supernatants were removed and cultures supplemented with fresh medium. In preliminary experiments we found that the enzymatic treatment does not influence the long-term *in vitro* survival of macrophages. Thus, at the end of culture cells were detached from wells at 4˚C with 0.02% EDTA solution in PBS and cell count as well as viability were estimated. The differences between untreated and collagenase-treated groups were insignificant and did not exceed ±10%.

Testicular macrophages (TMs) were isolated following a slightly modified procedure described by Bryniarski *et al.* [5]. Briefly, testicular capsule (tunica albuginea) was removed without cutting the tubules, and the interstitial tissue was gently loosened by 30G needles. Then testes were shaken in Mg^{2+} and Ca^{2+} free DPBS with 0.5% glucose at room temperature for 10 min at 100 cycles/min (non-enzymatic isolation, M-TMs). Alternatively, cells were treated with a mixture of 80 μM collagenase per 8 testes for 10 min at 37˚C in a shaking water bath. Then the enzymatic reaction was stopped by several washings of cells in DPBS supplemented with 20% FCS (collagenase isolation, E-TMs). Cells rescued from the interstitial tissue consist of TMs, Leydig cells and myoid cells.

Purification of TMs was performed by allowing the cells to adhere for 2 hrs at 37˚C to Petri dishes. Non-adherent cells were removed, and after thorough washing adherent cells were detached with 0.02% EDTA solution. This population contained 75-80% esterase- and FcγR-positive cells. Adherent cells were further purified by separation of FcγR-positive cells (rosetting cells) on the Lymphoprep. Adherent cells were mixed with TNP-substituted mouse erythrocytes (MRBC) opsonized with subagglutinating dilution of anti-TNP IgG2b mouse mAb and incubated for 30 min at 4˚C. Subsequently, cell mixture was layered over 10 ml of Lymphoprep, centrifuged at 500×g for 15 min at 4˚C, cells in the pellet were collected, MRBC were removed by osmotic shock [5] and TMs were used for estimation of cytokine production in cell culture. Alternatively, cells were purified by a second glass adherence to remove the traces of catalase from hemoglobin liberated from TNP-MRBC, which could interfere with the luminometric measurements of ROI's produced by macrophages. The final cell populations obtained by both procedures (*i.e.* double adherence or rosetting) contained over 95% of viable FcγR^{+} (see Table 1) and esterase^{+} cells [17-18].

Lucigenin-dependent chemiluminescence (LGC). Macrophages were cultured overnight at the concentration of 5×10^{5} viable cells/well in 0.2 ml RPMI 1640 medium supplemented with 10% FCS in 96-well flat bottom dark plates (Nunc, Roskilde, Denmark). After that 10 μM lucigenin was added and the cells were incubated for 15 min at 37˚C in a dark adaptation chamber [10]. Then in some groups macrophages were stimulated by addition of zymosan opsonized with mouse serum at a ratio 10 particles per cell and plates were immediately transferred to a Lucy 1 luminometer (Anthos, Salzburg, Austria). The photon emission was measured for 75-100 min. Each experiment was run in duplicate.

Cytokine immunoassay. Untreated or enzyme-treated TMs or PMs were cultured in 24-well flat bottom plates at a concentration of 5×10^{5}/ml in RPMI 1640 medium supplemented with 5% FCS. Supernatants for TNF-α estimation were collected after 24 hrs, and for IL-6, IL-10 and IL-12 after 48 hrs of culture and frozen at -80˚C until further use. Concentrations of IL-6, IL-12 and TNF-α in culture supernatants were measured in capture ELISA test, using 96-well flat Corning Easy Wash plates (Corning, Corning, NY). IL-10 was determined by using IL-10 OptEIA^{TM} ELISA set as recommended by manufacturer. Details of the procedure were published earlier [6]. The optical density of each well was measured at 492 nm in 96-well plate reader and concentrations of cytokines in samples were read from a standard curve. Recombinant murine cytokines were used as standards. Sensitivity of Elisa tests: IL-6, 15pg/ml; IL-10, 15pg/ml; IL-12 p40, 15pg/ml; TNF-α, 10pg/ml [6].

Statistics. The two-tailed Student's t-test was used to evaluate the statistical significance of experimental differences between groups with $p<0.05$ assumed as the minimum level of significance.

Results and discussion

Production of oxygen intermediates by enzyme-treated macrophages

TMs, irrespective of whether isolated by mechanical procedures or by enzymatic treatment, had a very limited capability to produce ROIs, and stimulation with zymosan had little additional effect. In contrast, PMs produced copious amounts of ROIs in the presence of zymosan, and short treatment with collagenase caused an 95% inhibition of radical production in comparison with untreated cells, the effect being not due to decreased cell viability. Figure 1 shows the results from one representative experiment out of three.

Since TMs are unable to produce significant amounts of ROIs spontaneously or after stimulation with zymosan, it indicates profound functional differences between these two macrophage populations with different location despite their common blood-borne origin. The lack of production of ROIs by TMs upon proper stimulation with zymosan may be due to two mechanisms:

(1) Lack of recognition. It is commonly accepted that phagocytosis of opsonized zymosan particles by macrophages depends primarily on integrity of CR2 but also FcR or mannose receptors. This mechanism in the case of TMs is unlikely since they have identical receptors as peritoneal macrophages. Moreover, as shown by us previously, short collagenase treatment does not affect these receptors [6].

(2) Lack of proper cellular machinery to react. It has been shown previously that enzymes, particularly

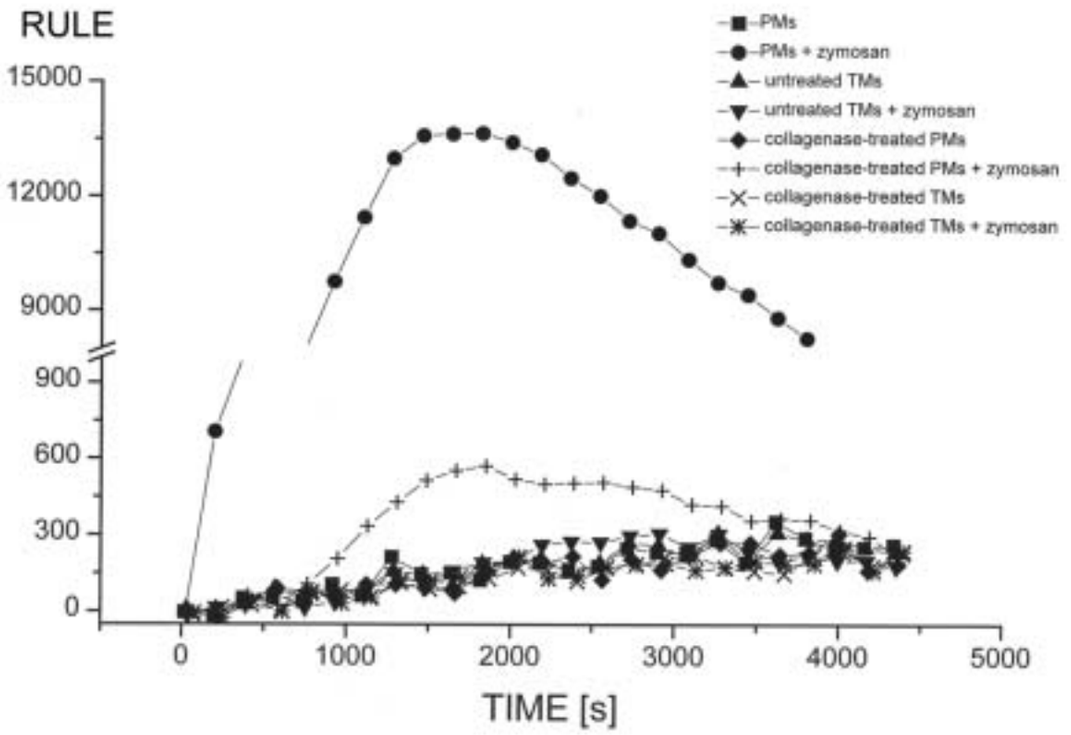

Fig. 1. Decreased production of reactive oxygen intermediates (ROIs) by peritoneal macrophages and testicular macrophages treated with collagenase. Testicular macrophages (TMs) and oil-induced peritoneal macrophages (PMs) were treated with 80 μM collagenase for 10 min or left untreated. Then to 5×10^5 cells in 0.15 ml nutrient medium lucigenin and zymosan were added and photon emission was measured over 75-100 min (abscissa, for details see Materials and methods). The results of one out of three experiments are demonstrated. Chemiluminescence (ordinate) is expressed in arbitrary units (RUL).

metalloproteases, released by phagocytes cleave many cell membrane molecules [6]. That mechanism may be responsible for diminished ROI synthesis by enzyme-treated PMs found in our experiments. NADPH oxidase catalysing the generation of ROIs, is composed of several cytosolic and membrane bound proteins which in resting phagocytic cells are segregated. After the cell receives a proper signal (*e.g.* phagocytosis), cytosolic components translocate to the cell periphery and anchor to membrane-bound proteins, flavocytochrome B complex, to form an active enzyme [8, 19]. We regard as a possible explanation that the treatment of PMs with proteolytic enzymes causes removal and/or functional impairment of cell surface-bound docking proteins which by impeding the formation of active enzymatic NADPH oxidase complex leads to diminished ROI production. In view of diminished capability of TMs to produce ROIs it would be interesting to test the NADH oxidase system in these cells.

The observed differences between TMs and PMs seem to have biological sense. TMs are located in the vicinity of seminiferous tubules containing spermatozoa and ROIs have a strong mutagenic influence on the genetic material. Under normal conditions, TMs produce little, if any, ROIs and even when stimulated with zymosan (this is a model of inflammatory stimulus) they remain indifferent. Similarly, as shown previously, TMs do not produce other radicals like nitrogen oxide which insures the integrity of neighbouring DNA [11].

Production of cytokines by collagenase-modified macrophages

The analysis of cytokine profile secretion of mechanically-isolated TMs shows that these cells are low active cytokine producers in comparison to PMs. Collagenase treatment affected significantly the production of cytokines by testicular macrophages (TMs) as well as oil-induced peritoneal macrophages (PMs). Results of one representative experiment out of three are shown in Table 1. Collagenase treatment of TMs induced a significant 13-fold increase in IL-6 production, 6-fold increase in TNF-α and 8-fold increase in IL-10 production in comparison to untreated cells. Enzymatic modification of PMs produced less spectacular effects - 2.5-fold increase in IL-6 as well as IL-10 synthesis and only slightly stimulated TNF-α production. The effect of

Table 1. The influence of collegenase treatment on the secretory activity of testicular and peritoneal macrophages

Group of macrophages	TNF-α [pg/ml]	IL-6 [pg/ml]	IL-10 [pg/ml]	IL-12 [pg/ml]	Rosette formation [%]
Testicular macrophages isolated by shaking	21±3	126±7	<15	17±1	96±1
Testicular macrophages collagenase-isolated	*132±12	*1661±15	*128±10	<15	95±2
Peritoneal macrophages	245±5	283±7	61±3	213±3	98±2
Peritoneal macrophages treated with collagenase	375±8	*705±12	*150±4	271±6	97±3

Five $\times 10^5$ untreated or collagenase-treated (80 μM) oil-induced peritoneal macrophages or testicular macrophages purified mechanically or by enzymatic treatment and separated according to rosette formation procedure (for details see Legend to Figure 1 and also Materials and methods) were cultured in 1 ml of RPMI 1640 medium for 24 hrs (for TNF-α) or 48 hrs and concentration of interleukin (IL-6, IL-10 and IL-12) in supernatants was measured by ELISA assay. Table shows the results of one representative experiment out of three. Results are expressed as the mean of three estimations ±SD Statistical significance $p<0.01$ between the relative groups (collagenase-treated against control) is marked by asterisks.

treatment of TMs and PMs with collagenase on IL-12 synthesis was negligible, although TMs irrespectively of the mode of separation produced IL-12 in concentration at the border of assay sensitivity in comparison to moderate amounts of IL-12 released by PMs. The relatively lower increase in cytokine production by PMs after enzymatic treatment in comparison to similarly treated TMs may be due to the fact that these cells are already activated by intraperitoneal stimulation with mineral oil. These enzyme-dependent mechanisms seem to be highly effective, since even short treatment of TMs with minute concentrations of the enzyme produced significant changes in cytokine production, and the effects were long lasting and could be observed even after 48 hrs in culture (results not shown).

The intense production of cytokines such as IL-6 and TNF-α shows that collagenase treatment of macrophages, both TMs and PMs, is recognised as a signal that turns on the cell machinery to release pro-inflammatory products. Simultaneously, however, anti-inflammatory regulatory mechanisms are put in motion - enhanced production of acute phase proteins, ACTH and corticosteroids as the result of IL-6 activity [1, 2, 16] and increased IL-10 synthesis. The mechanisms by which enzymatic treatment of cell surface triggers cytokine production are not clear at present, but there is some specificity in enzyme action, since synthesis of some cytokines (*e.g.* IL-12) remains unchanged [3]. This problem certainly demands further studies.

Enhancement of IL-10 secretion by collagenase with simultaneous lack of influence on IL-12 synthesis suggests that in the case of local inflammation TMs are poised to stimulate the local humoral response and to inhibit Th1 cellular immune responses. Those results conform to our previous observations which demonstrated that TMs are efficient antigen presenting cells in humoral response *in vitro*, but produce tolerance when used to induce cell-mediated immunity [12].

In summary, our experiments show that testicular macrophages represent a highly specialised cell population which in contrast to macrophages in other locations does not produce ROIs upon pro-inflammatory stimulation and in effect prevents the possible damage to genetic material. Moreover, TMs when exposed to inflammatory signals like proteolytic enzymes produce an array of cytokines which may by feedback induce anti-inflammatory mechanisms (IL-6). Under these circumstances, highly increased production of IL-10 directs the possible immune responses into humoral pathway, thus preventing the development of the cell-mediated responses, deleterious to testicular tissues.

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